Biotransformation of Organic Residues to Useful Materials by Burkholderia cepacia(Dissertation_全文)

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Contents

Introduction

The bacterial strain used in this study was isolated from benzalkonium chloride solution. It was identified as *Burkholderia cepacia* according to Bergy's manual at The Research Foundation for Microbial Disease of Osaka University. It is now preserved as IFO No.15124 strain at the Institute for Fermentation Osaka.

In the field of manufacturing the sterilized products, such as drugs like eye drops and toiletries like shampoo, pollution problems occur with the bacteria (*Burkholderia cepacia*). On the other hand, clinically the bacteria causes opportunistic infection that is troublesome for medical doctors.

Benzalkonium chloride and *p*-hydroxybenzoates (parabens) are known as representative antiseptics and disinfectants. The isolated strain is resistant to these antiseptics and disinfectants, generally toxic compounds, using to inhibit multiplication of pollutant bacteria. In this point of view, it was assumed that the strain (IFO No.15124) could degrade organic residues and we would examine biotransformation of organic residues to useful materials.

Parabens were completely degraded in three weeks by the strain. We isolated and identified *p*-hydroxybenzoic acid and protocatechuic acid as metabolites.

The strain, one of the environmental bacteria, need quite a little nutrients for living and can live for long time even in pure water. It is known, however, *Burkholderia cepacia* are sterilized by heating at 45℃ for an hour and can not live in over 10% methanol water solution. So we can control the hazard caused by them.

Standard experimental procedures are as follows. An appropriate concentration saline solution of the substrate was prepared. A few loops of the bacteria subcultured on SCD (soybean casein digest) agar medium were inoculated into the solution and allowed to stand at 30℃. The concentration of the substrate was monitored by means of HPLC. About the chemicals the concentration of which decreased, we isolated the metabolite and identified the structure by means of IR and MS comparing with authentic sample.

Organic residues and metabolites transformed by the strain are described below.

1) Methylparaben, ethylparaben, *n*-propylparaben, *n*-butylparaben were degraded through *p*-hydroxybenzoic acid and protocatechuic acid.
2) *p*-Hydroxybenzoic acid was degraded through protocatechuic acid.
3) *m*-Hydroxybenzoic acid was degraded through protocatechuic acid.
4) Salicylic acid was degraded through 2,3-dihydroxybenzoic acid.
5) Benzoic acid was degraded through salicylic acid.
6) *p*-Cresol was degraded through 4-methylcatechol.
7) *m*-Cresol was degraded through 4-methylcatechol.
8) *o*-Cresol was degraded through 3-methylcatechol.
9) *p*-Nitrophenol was degraded through 4-nitrocatechol.
10) *m*-Nitrophenol was degraded through 4-nitrocatechol.
11) *p*-Hydroxybenzaldehyde was degraded through *p*-hydroxybenzoic acid.
12) Phenol was degraded through pyrocatechol and pyrogallol.
13) Pyrocatechol was degraded through pyrogallol.
14) 1-Naphthol was degraded through 1,2-dihydroxynaphthalene.
15) 2-Naphthol was degraded through 1,2-dihydroxynaphthalene.
16) Phenacetin was degraded through *p*-phenetidine.
17) Hippuric acid was degraded through benzoic acid.
18) Phenaceturic acid was degraded through phenylacetic acid.
19) Oxindole was degraded through isatin.
20) Benzimidazole was degraded through 5-hydroxybenzimidazole.
21) Polysolbate 80 was degraded with the formation of oleic acid.
22) *p*-Phenetidine, anthranilic acid, 1-naphthylamine, 2-naphthylamine, *p*-phenylphenol, *o*-aminobiphenyl, *p*-aminobiphenyl, 3-indoleacetic acid, isatin, stilbesterol, aspartame, caffeic acid, benzonitrile, benzoylformic acid, *p*-acetamidephenol, mandelonitrile, 2-hydroxyphenylethylamine, styreneglycol acetate, styreneglycol propionate, tetramethylthiuram disulfide were degraded. (metabolites were unknown).
23) Indole was oxidized rapidly to oxindole and then degraded.
24) Skatole was oxidized rapidly to 2,3-dihydroxyskatole and then degraded. The bacteria was assumed to be able to use as a biodeodorant for indole and skatole. Indole and skatole were oxidized rapidly to be odorless by the bacteria. Conversion ratio of indole to oxindole was near 10%, and many side reactions were assumed to occur. Purple precipitate was observed in the culture liquid. It was insoluble in acid, alkali and ethyl acetate, and was soluble, however, in methanol in time. Many colorful pigments were

separated by TLC.

25) Carbaryl, an insecticide, was degraded through 1-naphthol.

As described above, it was clarified that the strain degraded many organic residues. To control the reaction, we tried to transform organic residues to useful materials.

There have been many reports on microbial hydroxylations of aromatic compounds. Though the hydroxylations of aliphatic compounds by microorganisms are highly evaluated in the field of organic synthesis, that of aromatic compounds has been regarded as worthless. One of the main reasons is that introducing hydroxyl groups into benzene rings causes distortion of aromaticity, and the oxidation products obtained turn out to be susceptible to other attacks by microorganisms and finally to degradation. Another reason is that most of the previous papers were concerned with the pathways of the oxidative degradation of aromatic compounds in the field of biology and biochemistry and not with the production of useful oxidized products by using microorganisms as biocatalysts.

Benzyl alcohol, however, was biotransformed to salicyl alcohol stoichiometrically. Salicyl alcohol was accumulated because of relatively little further degradation.

Salicyl alcohol is local anesthetic itself and an important starting material for organic synthesis.

According to 2,3,4,6-tetra-*O*-acetyl- β -D-glucopyranosyl isothiocyanate (GITC) derived prelabeled HPLC, to separate D-type and L-type of amino acids, we examined D or L-recognition of them by the strain. We could easily gain the experimental results by adopting culture system using simple substrate saline solution. L-Selective digestion proceeded in the cases of tyrosine, phenylalanine, alanine, proline, histidine, serine and threonine. While D-selective digestion proceeded in the cases of valine, leucine and isoleucine.

D-Tryptophan was specifically accumulated by L-specific digestion of tryptophan by the strain.

We investigated enantiospecific recognition of the optically active

compounds by the strain. The analysis of the experimental results were performed by HPLC using a Shiseido Ceramospher Chiral RU-1 column, commonly used for the separation of *rac*-compounds. The results obtained by the experiments are as follows; *rac*-1-phenyl-2-propyl acetate was hydrolyzed (*R*)-selectively to (*R*)-(-)-1-phenyl-2-propanol and (*S*)-(+)-1-phenyl-2-propyl acetate by the strain. *Rac*-1-phenyl-1-ethyl propionate was hydrolyzed (*R*)-selectively to (*R*)-(+)-1-phenyl-1-ethanol and (*S*)-(-)-1-phenyl-1-ethyl propionate by the strain.

We obtained the following further experimental results; the reaction efficiencies were sometimes increased by immobilization of the strain.

Encapsulated cells of the strain by polyacrylamide degraded carbaryl rapidly than the case of free cells. Furthermore, second-used encapsulated cells also degraded carbaryl rapidly than the case of free cells. One of the reasons was estimated that the adsorption of 1-naphthol on the polyacrylamide gel occurred, and 1-naphthol itself also degraded rapidly. Therefore, the reaction velocity of the substrate with encapsulated cells was accelerated by the consumption of 1-naphthol.

We clarified the bacteria catalyzed transesterification in the presence of alcohol. The strain catalyzed transesterification of substrates adding excess alcohol. The higher the content of alcohol was added, the higher the proceeded conversion ratios appeared, because slower side reactions occurring.

The strain could live in 5% methanol and could not live in 10% methanol. Immobilizing the strain by alginic acid, however, the bacteria was not inactivated even in 30% methanol. It is noteworthy that using immobilized cells, we could use higher contents of alcohol and could attain higher conversion ratio.

There are many reports about biotransformation by *Burkholderia cepacia*.

Regarding degradation of organic residues, many papers concern with chloroorganic compounds oxidation. Especially, degrading 2,4-dichlorophenoxyacetic acid (2,4-D)[1] and 2,4,5-trichlorophenoxyacetic acid

(2,4,5-T)[2] are examined deeply and widely. The strain used in this experiment, however, did not degrade them. Degradation of polychlorinated biphenyls (PCBs)[3] and trichloroethylene (TCE)[4] are investigated vigorously using the bacteria. About toluene degradation, there are two types of pathway. One is 2,3-dioxidation by 2,3-dioxygenase[5], and another is *ortho*-monooxidation by *ortho*-monooxydase[6]. The strain used in this experiment product only *ortho*-monooxydase. Further degradation is cleavage of benzene ring in either case.

Regarding production of useful materials, many papers concern with stereoselective resolution[7]. It is noteworthy that the bacteria product antifungal compounds such as pyrrolnitrin[8] and a novel lipopeptide[9].

This paper consists of five chapters. In chapter 1, we discuss biotransformation activity of the bacteria on *p*-hydroxybenzoates and benzalkonium chloride. In chapter 2, we discuss encapsulated cells of carbaryl and hippuric acid-degrading bacteria in polyacrylamide. In chapter 3, we discuss biotransformation of benzyl alcohol by the bacteria. In chapter 4, we discuss transesterification of ethylparaben by free and entrapped cells of the bacteria. In chapter 5, we discuss enantiomeric resolution of 1-phenyl-2-propanol by the bacteria.

Chapter 1

Biotransformation Activity of *Burkholderia cepacia* on *p*-Hydroxybenzoates and Benzalkonium chloride

A bacterial strain, which was isolated from 10% benzalkonium chloride solution and identified as *Burkholderia cepacia*, grew in a saline solution of *p*-hydroxybenzoates (100ppm), commonly called parabens and used as an antiseptic. Methyl, ethyl, *n*-propyl and *n*-butyl *p*-hydroxybenzoates were almost completely degraded by *Burkholderia cepacia* after three weeks of culturing. After two weeks of culturing, *p*-hydroxybenzoic acid was identified as metabolite of parabens, showing the bacteria's ability to cleave the C-O linkage in ester bonds. On the other hand, benzalkonium chloride, a typical antiseptic, was not metabolized but the bacteria continued to live throughout the experiment, thus showing its capacity to resist benzalkonium chloride.

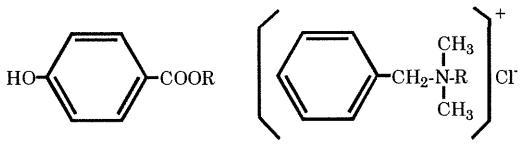
Introduction

It has become a serious problem in the manufacturing of drugs, cosmetics and foods that some bacteria are resistant to antiseptic, which are expected to control the bacterial growth. There have been many reports on the mechanism of bacterial resistance to antibiotics and disinfectants[10]. However, most of them have been investigations on the morphological changes or change of components in resistant bacterial cells[10].

Many reports are available on the biotransformation of organic compounds by the catalytic action of *Burkholderia* sp.[11-13]. Some of them, however, were carried out by using complicated artificial culture medium or buffer solutions during cultivation, which leaves some disadvantageous problem on the analyses of metabolites.

This study has been attempted to clarify the degradation of some *p*-hydroxybenzoates, commonly called parabens and used in drugs and cosmetics as an antiseptic, by the bacterium. This chapter reports on the biotransformation of methyl, ethyl, *n*-propyl and *n*-butyl *p*-hydroxybenzoates (Fig.1-1(1)-(4)) by the catalytic action of the bacterium, namely its ability to cleave to the C-O linkage in ester bonds. In addition, this chapter describes the bacterial action in reference to benzalkonium chloride (Fig.1-1(5)),

another commonly used antiseptic.



(1) methyl *p*-hydroxybenzoates R=CH_3
(2) ethyl *p*-hydroxybenzoates R=C_2H_5
(3) *n*-propyl *p*-hydroxybenzoates R=C_3H_7
(4) *n*-butyl *p*-hydroxybenzoates R=C_4H_9
(5) benzalkonium chloride R=$C_{14}H_{29}$

Fig. 1-1 Structures of *p*-hydroxybenzoates (A) and benzalkonium chloride (B).

Materials and Methods

1. Bacterium

The bacterium used in this experiment was isolated from 10% benzalkonium chloride solution as follows. The solution was filtered by a membrane filter, the filter was washed with sterilized water and placed on SCD (soybean casein digest) agar medium at 30℃ for two days. The colonies formed were further acclimatized to the same medium and these procedures were repeated several times. The bacterial strains isolated were identified as *Burkholderia cepacia* according to Bergy's manual at The Research Foundation for Microbial Disease of Osaka University and strain No-1 was used in this study.

2. Chemicals and experimental equipments

p-Hydroxybenzoates, *p*-hydroxybenzoic acid and benzalkonium chloride were purchased from Nacalai Tesque. IR : HITACHI 260-10. MS : HITACHI MS-80B. HPLC : SHIMADZU LC-6A.

3. Culture and administration method

The sample was dissolved in a saline solution in concentration of 100ppm, which was used as a medium. 500ml Erlenmeyer flasks containing 250ml of the medium were autoclaved for 20min. A few loops of bacteria, subcultured on SCD agar medium, were inoculated into flasks, which were allowed to stand for one to three weeks at 30℃.

4. Performance of HPLC

In the mobile phase, 0.01M $NH_4H_2PO_4$-CH_3CN (7:3)(adjusted pH to 2.5 with H_3PO_4) for the analysis of *p*-hydroxybenzoates, and 3:7 portions of the same reagent for analysis of benzalkonium chloride were used. The column was Shiseido Capcell Pak C_{18} SG-120, commercially packed with reversed-phase octadecylsilica (150mm×6.0mm I.D.), through which the above mobile phase was run at a flow-rate of 1.0ml/min. For analysis of *p*-hydroxybenzoates, operation was carried out at 254nm and for that of benzalkonium chloride at 210nm. Samples of 10 μ l were applied on the column. The concentrations of compounds were calculated according to the internal standard method.

5. Isolation and identification of the conversion products from *p*-hydroxybenzoates

After culturing for one to three weeks, an aliquot of the culture filtrate was directly subjected to HPLC analysis. The isolation and identification of metabolites in the case of ethyl *p*-hydroxybenzoates were as follows. After culturing for two weeks, culture filtrate (2.5L) was acidified with 2N-HCl and extracted with ethyl acetate. Evaporation of the solvent gave light yellow materials (79mg), which were separated into six parts by the preparative TLC (Merck, Art 5715 Kieselgel 60 F_{254}) with a solvent system of $CHCl_3$-methanol-acetic acid (90:10:1), and the metabolites were detected through U.V. radiation. By this procedure, well-separated six bands were collected and gave part 1 (R_f : 0.95, 1.4mg), part 2 (R_f : 0.80, 20.0mg), part 3 (R_f : 0.50, 1.1mg), part 4 (R_f : 0.40, 56mg), part 5 (R_f : 0.18, 1.1mg) and part 6 (R_f : 0.0, 1.0mg), respectively.

Results and Discussion

After culturing for three weeks, all *p*-hydroxybenzoates were found to be almost completely degraded as shown in Fig.1-2.

The time course of degradation of the compounds tested was almost the same, and we tried to identify the metabolites of these compounds.

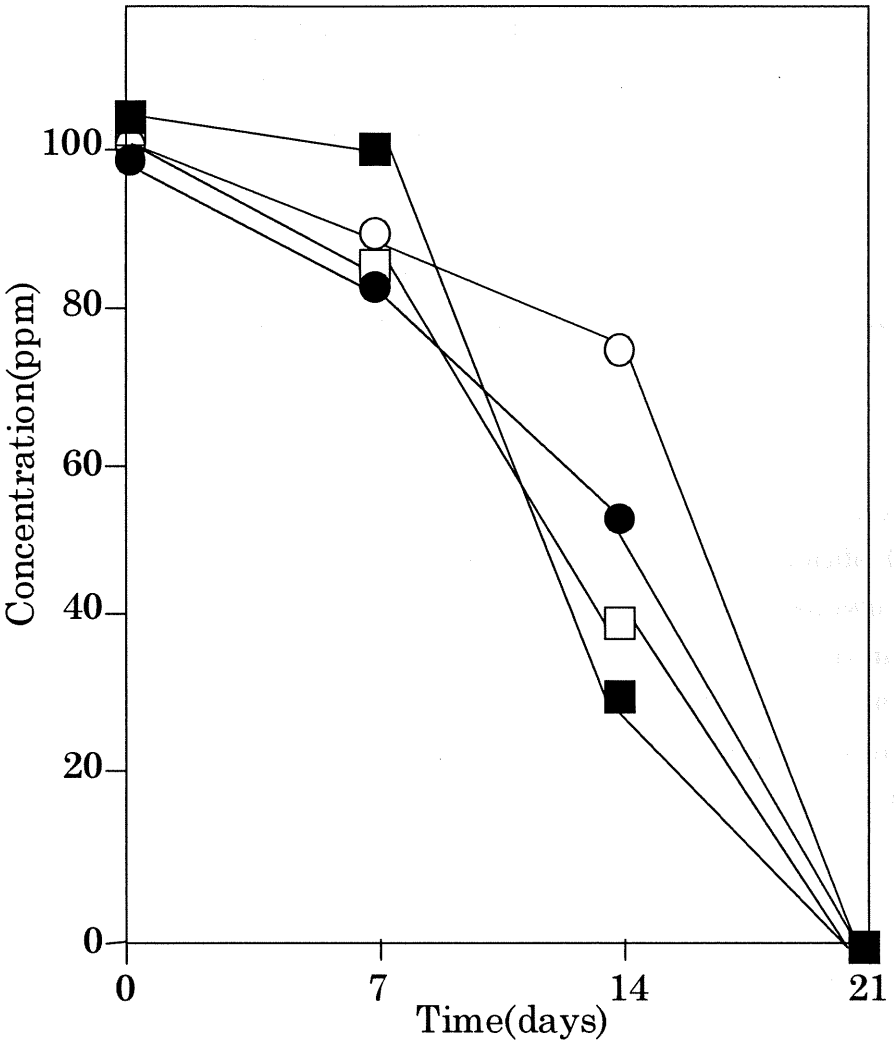


Fig.1-2 The degree of degradation of *p*-hydroxybenzoates with time:
(○) (1) methyl *p*-hydroxybenzoate; (●) (2) ethyl *p*-hydroxybenzoate;
(□) (3) *n*-propyl *p*-hydroxybenzoate; (■) (4) *n*-butyl *p*-hydroxybenzoate.

1. Identification of *p*-hydroxybenzoic acid

The part 4 compound obtained from the degradation experiment of ethyl *p*-hydroxybenzoate was recrystallized from methanol to colorless needles (43mg) of m.p. 212-214℃ (lit.[14] 213-214℃). IR_{max} (KBr) cm^{-1} : 3300-3500 (OH), 1680 (dimer of acid), 1615 (phenyl), 1600 (phenyl), 1510 (phenyl), 1425 (OH), 855 (*p*-substituted phenyl); MS (EI) m/z : 138 (M^+, $C_7H_6O_3$), 121 (M-OH)$^+$, 93 (121-CO)$^+$, 65 (93-CO)$^+$.

From these results, the part 4 compound was presumed to be *p*-hydroxybenzoic acid, and was proved to be so by comparing its IR and mass spectra with those of an authentic compound.

In the case of *p*-hydroxybenzoates other than ethyl ester, *p*-

hydroxybenzoic acid was obtained in the yields of 12.5mg (methyl ester), 3.0mg (*n*-propyl ester) and 2.5mg (*n*-butyl ester), respectively.

We did not take metabolites, *p*-hydroxybenzoic acid and others, into consideration when we discuss the action of the bacterium on *p*-hydroxybenzoates, because those metabolites were quantitatively too small to be considered and be degraded promptly.

Summarizing the information obtained in the experiment, the bacterium is able to hydrolyze the C-O linkage of parabens.

2. Metabolism of benzalkonium chloride by the bacterium.

We also investigated the degradation of benzalkonium chloride by the bacteria. Contrary to our expectation, benzalkonium chloride (ca. 50ppm) was left unchanged after culturing for four weeks as shown in Fig.1-3. However, it was noteworthy to find that the bacterium remained alive throughout the experiment as shown in a growth-test of bacteria on agar medium (data not shown). This phenomenon is of interest in connection with the structural differences between *p*-hydroxybenzoates and benzalkonium chloride.

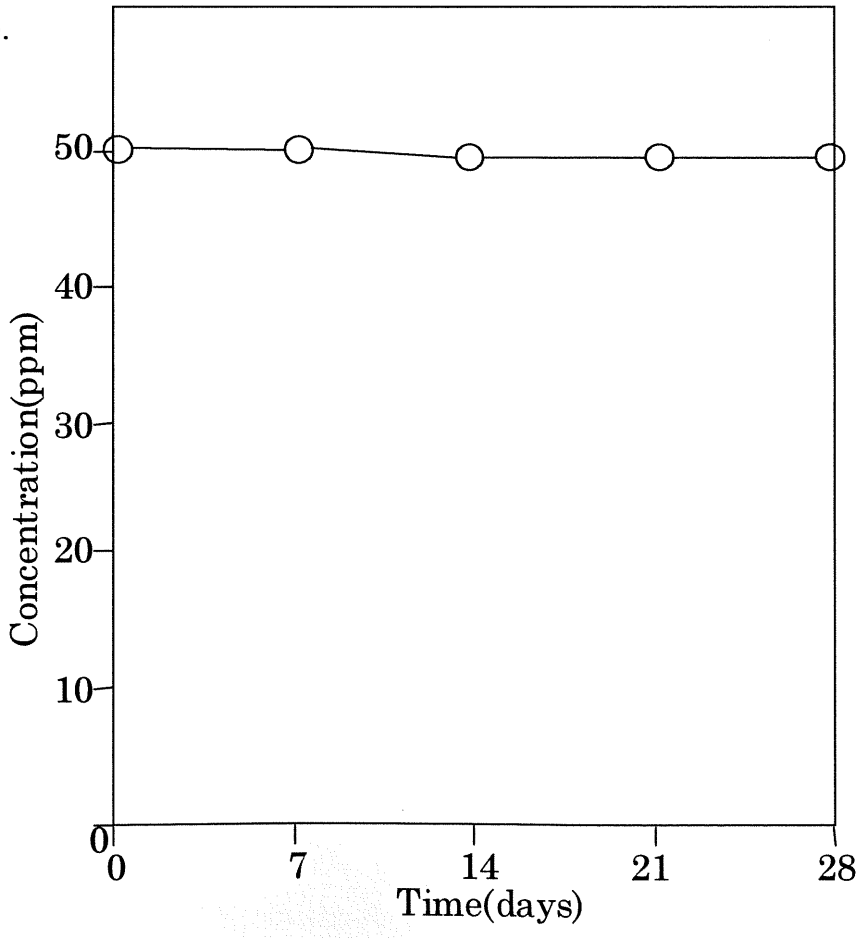


Fig.1-3 The degree of degradation of benzalkonium chloride with time.

Conclusion

The results obtained in this study indicated that the bacterium utilized *p*-hydroxybenzoates commonly added as antiseptics as the sole carbon source. After two weeks of culturing, *p*-hydroxybenzoic acid was identified as metabolite of *p*-hydroxybenzoates. After three weeks of culturing, *p*-hydroxybenzoates were almost completely degraded.

On the other hand, benzalkonium chloride, a typical antiseptic, was not metabolized after four weeks of culturing. However, the bacteria remained alive throughout the experiment, showing its strong capacity to resist benzalkonium chloride.

Chapter 2

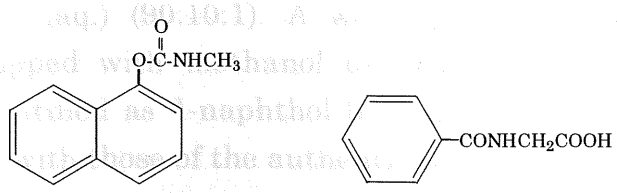
Encapsulated Cells of Carbaryl and Hippuric Acid-Degrading Bacterium *Burkholderia cepacia* in Polyacrylamide

In the course of our investigation on the biotransformation of organic compounds by *Burkholderia cepacia*, we have reported that representative antiseptics parabens, *e.g.*, methyl, ethyl, *n*-propyl and *n*-butyl *p*-hydroxybenzoates, were almost completely degraded by the bacterium after 3weeks of culturing and *p*-hydroxybenzoic acid was identified as metabolite[15].

Mankind faces many serious environmental pollution problems. The environmental fate of agricultural chemicals is of great concern because some of them might be the cause of environmental pollution. For instance, effluence from farms or golf courses involves us all. This chapter has been attempted to degrade such chemicals using encapsulated cells of bacteria. We examined the biotransformation activity of *Burkholderia cepacia* on carbaryl, 1-naphthyl methylcarbamate, one of the most commonly used agricultural chemicals.

Some reports are available on the encapsulation of microorganisms on the solid supports[16,17]. In this chapter, commonly used polyacrylamide was selected and examined because other solid supports such as sodium alginate, urethane prepolymers PU-3, PU-6 and ENT were found to be unsuitable because of the leak of bacterium from the gel.

On the other hand, considering acylase activity of the bacterium, hippuric acid having an amide group in it was selected and examined whether it was degraded by the bacterium. In the cases of both carbaryl and hippuric acids, the reaction velocity of encapsulated cells and free cells with them were compared and investigated (Fig.2-1).



Carbaryl Hippuric acid

Fig.2-1 Structure of carbaryl and hippuric acid.

Materials and Methods

The bacterium used in this experiment was given from Institute for Fermentation (Osaka), strain 15124. Commercially available carbaryl, hippuric acid and some chemicals for polymerization of acrylamide were used.

1. Culture and administration of substrates

A 10ppm saline solution of carbaryl and hippuric acid was used as a culture medium. In each case, a number of 500ml Erlenmeyer flasks containing 250ml saline solution were autoclaved for 20min at 2.0bar and 120℃, to which carbaryl or hippuric acid was added. A few loops of bacteria, subcultured on SCD agar medium, were inoculated into flasks, which were allowed to stand for 1-5weeks at 30℃.

2. Performance of HPLC

As the mobile phase, 0.01M $NH_4H_2PO_4$ (adjusted to pH 2.5 with H_3PO_4) - CH_3CN (6:4 for carbaryl, 15:85 for hippuric acid) was used. The column used was Shiseido Capcell Pak C_{18} SG-120 (150mm×6.0mm I.D.), commercially packed with reversed-phase octadecylsilica, through which the above mobile phase was run at a flow rate of 1.0ml/min. The operation was carried out according to the internal standard method.

3. Isolation and identification of the conversion product from carbaryl

Ten ppm saline solution of carbaryl (2.5L) was used as a substrate. After culturing for 14days, culture liquid was extracted with ethyl acetate. Evaporation of the solvent gave the dark purple materials, which were subjected to the preparative thin-layer chromatography (TLC) on silica gel (Merck, Art 5715 Kieselgel 60 F_{254}) with a solvent system of $CHCl_3$-methanol-ammonia (aq.) (90:10:1). A well-separated band, R_f 0.86, was collected and stripped with methanol to colorless materials (4mg). The substance was identified as 1-naphthol by comparison of its IR and mass spectroscopy (MS) with those of the authentic sample.

4. Isolation and identification of the conversion product from hippuric acid

Ten ppm saline solution of hippuric acid (2.5L) was used as a substrate.

After culturing for a week, culture liquid was extracted with ethyl acetate. Evaporation of the solvent gave colorless materials, which were subjected to the preparative TLC on silica gel (Merck, Art 5715 Kieselgel 60 F_{254}) with a solvent system of $CHCl_3$-methanol-acetic acid (90:10:1). A band, R_f 0.6, was collected and stripped with methanol to colorless materials (1.8mg). The 100ppm methanol solution of the substance was subjected to high-performance liquid chromatography (HPLC) analysis. When 10 μ l of the solution was injected onto the column under the conditions of HPLC mentioned above, the retention time (t_R 18.9) was obtained (benzoic acid t_R 19.1). Electron impact ms (EI-MS) of the substance showed m/z 122 (M^+, MW of benzoic acid 122) and the same fragment pattern as that of authentic benzoic acid.

5. Standard assay of biotransformation activities of encapsulated cells

The encapsulation of the bacterium was performed according to Chibata and Tosa's method[18]. The saline solution of carbaryl or hippuric acid (10ppm) was incubated with the encapsulated cells (7.5g wet weight) and allowed to stand for 1-4weeks at 30℃. As a comparative assay, the substrate solution with polyacrylamide gel (blank test) was also incubated and subjected to HPLC assay.

Results and Discussion

1. Degradation of carbaryl by free cells and identification of metabolite

After culturing for 25days, almost half quantity of carbaryl was degraded as shown in Fig.2-2. As shown in the experimental section, 1-naphthol was identified as a metabolite, showing the bacteria's ability to cleave the C-O linkage in ester bond of carbaryl.

2. Degradation of carbaryl by encapsulated cells

As shown in Fig.2-3, carbaryl was completely degraded within 2weeks. Interestingly, the degradation activity of bacteria was found to be remarkably increased after the encapsulation, showing that polyacrylamide is an efficient solid supporter in this case.

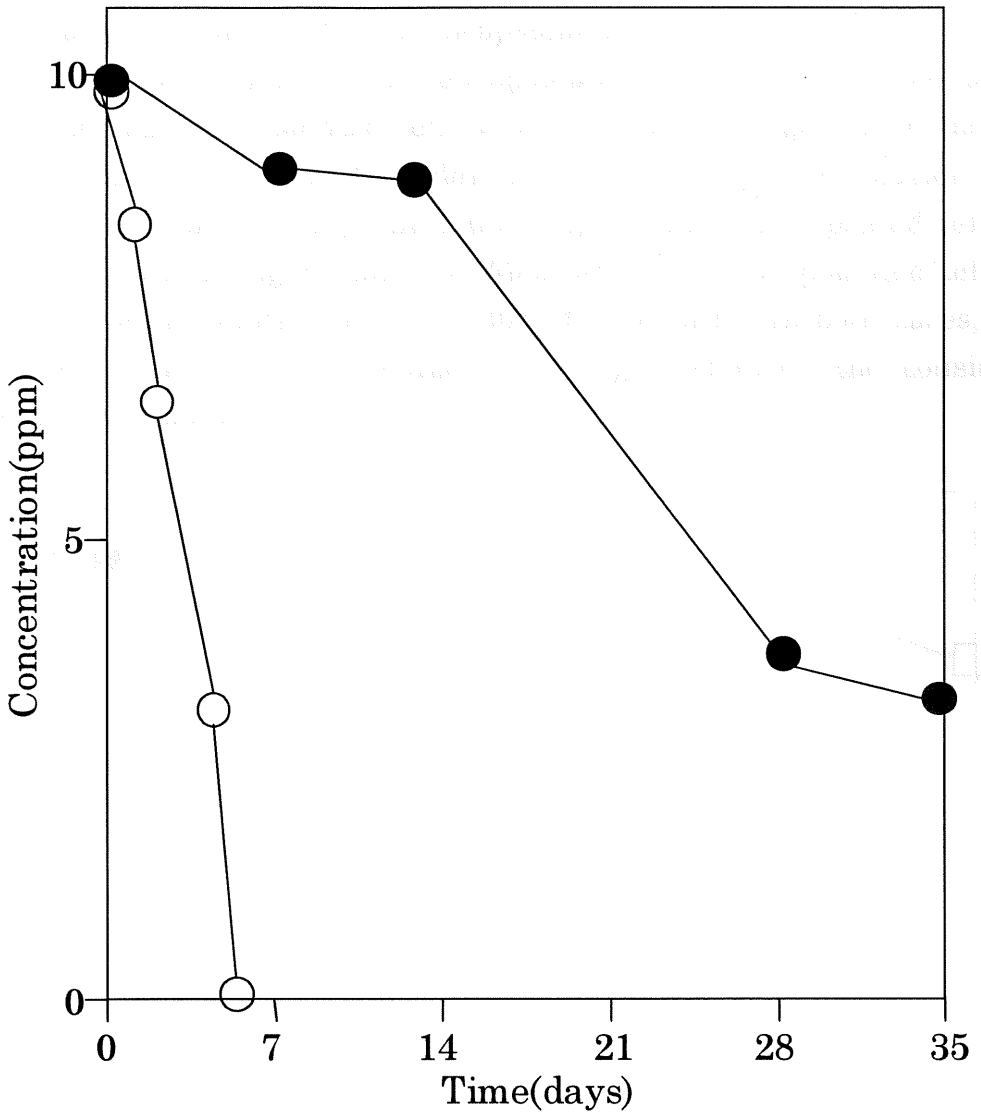


Fig.2-2 Degree of degradation of carbaryl and hippuric acid with time, using free cells: (●) carbaryl; (○) hippuric acid.

3. Degradation of hippuric acid by free and encapsulated cells and identification of metabolite

Hippuric acid was completely degraded for 6days' culturing as shown in Fig.2-2. As shown in the experimental section, benzoic acid was identified as a metabolite of hippuric acid, showing the bacterial ability to cleave the CONH linkage. Meanwhile, as shown in Fig.2-4, 9.89ppm of the initial concentration of hippuric acid was reduced to 3.41ppm after culturing for 12days. This fact shows that the activity was decreased a little after encapsulation.

4. Repeated use and stability of encapsulated cells

The relative activity of the encapsulated cells after repeated use was examined. The encapsulated cells were washed thoroughly with the saline solution each time before the addition of a new substrate solution. It was found that the activity remained unchanged when the repeated cells were used, as shown in Fig.2-3 and 2-4. Meanwhile, the encapsulated cells were stored in saline solution at 5 and 30℃ for 3months. In both cases, it was found that the activity remained unchanged, showing the considerable stability as a biocatalyst.

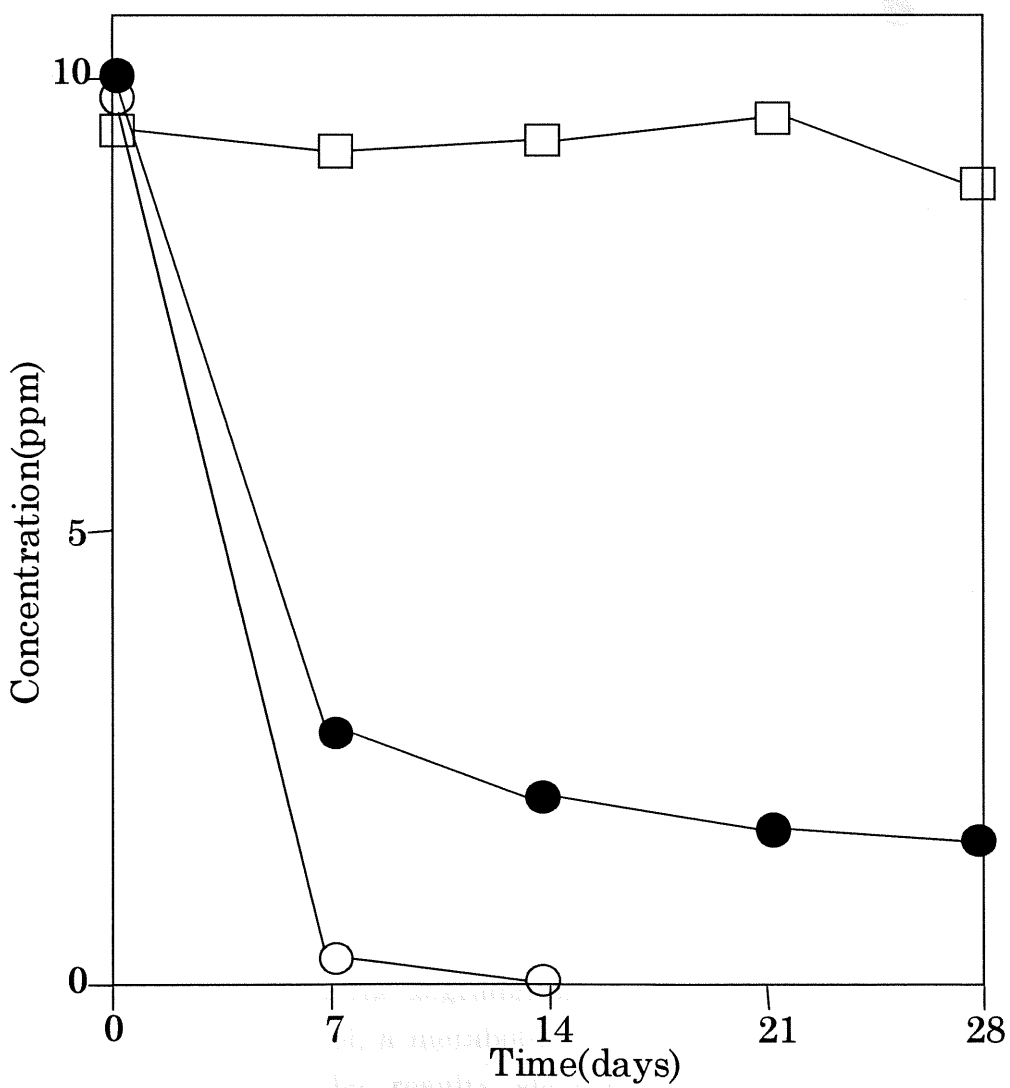


Fig.2-3 Comparative degree of degradation of carbaryl with time, using encapsulated cells, second-used encapsulated cells, and polyacrylamide gel: (○) encapsulated cells; (●) second-used encapsulated cells; (□) polyacrylamide gel.

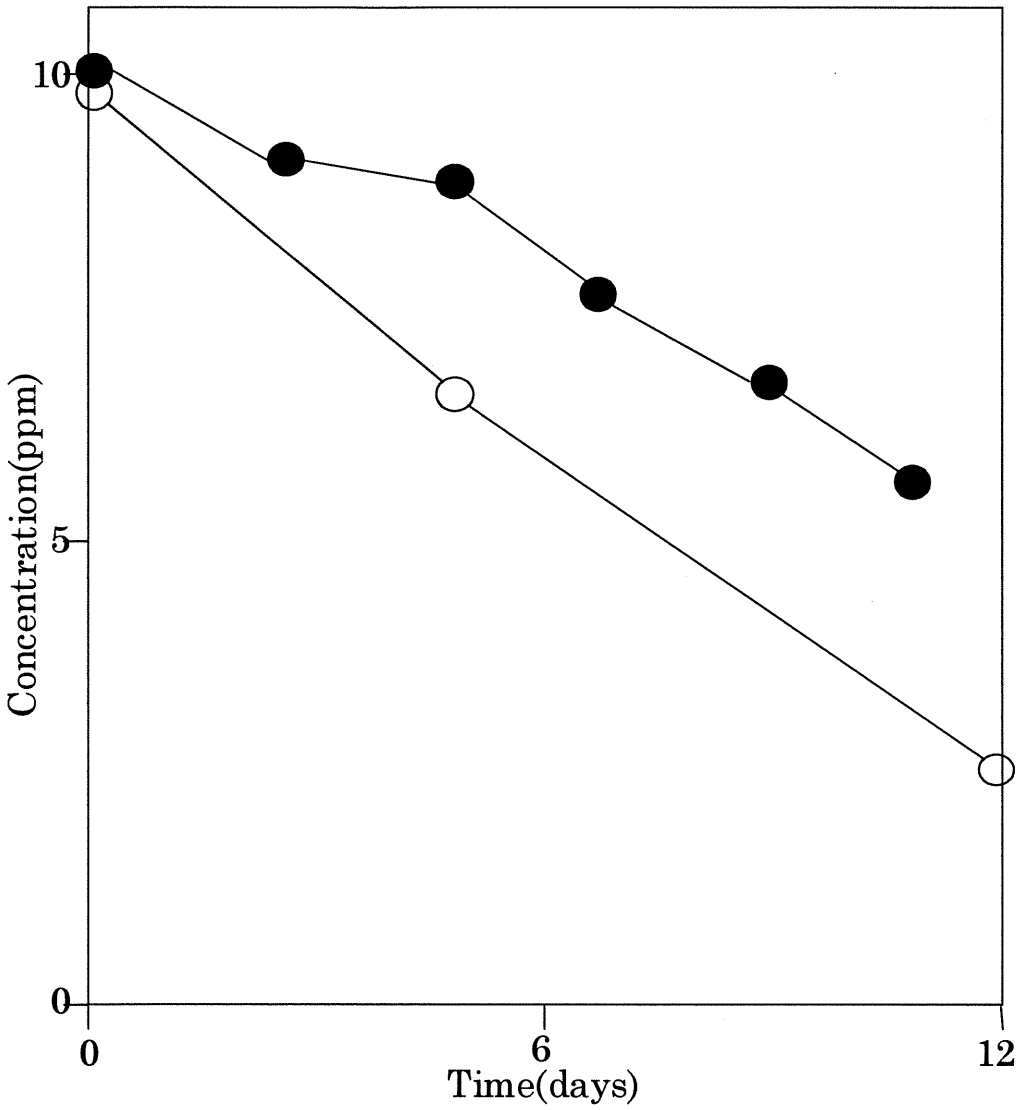


Fig.2-4 Comparative degree of degradation of hippuric acid with time, using encapsulated cells and second-used encapsulated cells: (○) hippuric acid; (●) hippuric acid (second-used encapsulated cells were used).

5. Consideration on effective activity of encapsulated cells concerning the degradation of carbaryl

As shown in Fig.2-3, the activity was remarkably increased after encapsulation concerning the degradation of carbaryl. To explore the reason, the reaction of 1-naphthol, a metabolite of carbaryl, with the encapsulated cells was examined. The results showed that 9.79ppm of the initial concentration of 1-naphthol was found to be reduced to 0.09ppm after culturing for a week. Interestingly, 9.34ppm of the initial concentration of the substrate was reduced to 4.01ppm after incubation for a week when

polyacrylamide gel was used instead of encapsulated cells (blank test). This phenomenon can be ascribable to the adsorption of 1-naphthol on the polyacrylamide gel. Therefore, the reaction velocity of the substrate with encapsulated cells was accelerated by the consumption of 1-naphthol.

Chapter 3

Biotransformation of Benzyl Alcohol by *Burkholderia cepacia*

There have been many reports on microbial hydroxylations of aromatic compounds[19]. Though the hydroxylations of aliphatic compounds by microorganisms are highly evaluated in the field of organic synthesis, that of aromatic compounds has been regarded as worthless. One of the main reasons is that introducing hydroxyl groups into benzene rings causes distortion of aromaticity, and the oxidation products obtained turn out to be susceptible to other attacks by microorganisms and finally to degradation. Another reason is that most of the previous papers were concerned with the pathways of the oxidative degradation of aromatic compounds in the field of biology and biochemistry and not with the production of useful oxidized products by using microorganisms as biocatalysts.

In the course of our investigation of biotransformation of organic compounds by *Burkholderia cepacia*, an interesting aquatic bacterium surviving for more than a year even in distilled water, we have already reported that the well-known antiseptics parabens, *e.g.*, methyl, ethyl, *n*-propyl and *n*-butyl *p*-hydroxybenzoates, were almost completely degraded by the bacterium after 3weeks of culturing and *p*-hydroxybenzoic acid was identified as a metabolite[15]. We have also reported that carbaryl, 1-naphthol methylcarbamate, one of the most commonly used agricultural chemicals, and hippuric acid having an amide group in it were degraded by the bacterium and 1-naphthol and benzoic acid were identified as metabolites, respectively[20]. In this chapter, we present the transformation of benzyl alcohol to salicyl alcohol by *Burkholderia cepacia* in the yield of about 30%.

Materials and Methods

1. The strain used in this experiment

The bacterium in this study was obtained from the Institute of Fermentation Osaka, Japan (IFO, IFO No.15124).

2. Experimental equipments

HPLC was done on a Shimadzu LC-6A liquid chromatograph with a UV

detector at 254nm. Mass spectra (MS) were recorded on a Hitachi 260-10 spectrometer.

3. Experimental procedures

Benzyl alcohol was dissolved in sterilized water at 1000ppm. In each case, a number of 500ml Erlenmeyer flasks containing 250ml of the solution were autoclaved for 20min. A few loops of the bacteria, subcultured on soybean casein digest agar medium (containing 15g of peptone, 5g of soybean-peptone, 5g of NaCl, and 15g agar/1 liter) for 2days at 30℃, were inoculated into flasks and were incubated statically at 30℃.

4. Performance of HPLC

For the analysis of benzyl alcohol using HPLC, the mobile phase, 0.01M $NH_4H_2PO_4$-CH_3CN (85:15)(pH adjusted to 2.5 with H_3PO_4), was used. The column used was a Shiseido Capcell Pak C_{18} SG-120, commercially packed with reversed-phase octadecylsilica (150mm×6.0mm I.D.), through which the above mobile phase was run at a flow-rate of 1.0ml/min. In each case, samples of 10 μ l were injected onto the column. The concentration of compounds was calculated according to the internal standard method. After culturing for definite times, culture liquid were directly analyzed by HPLC.

Results and Discussion

1. Degradation of benzyl alcohol

As shown in Fig.3-1, almost half of the benzyl alcohol was metabolized after the culture went on for a month. No change in the concentration of benzyl alcohol was observed under the same conditions in the blank test. The initial pH of culture liquid was about 7, and was unchanged during the incubation.

The OD_{660} of the culture liquid are shown in Fig.3-2. Numbers of the bacteria were found to increase gradually during the bioconversion period.

When phosphate buffer solutions were used as culture media, the results were similar to that above mentioned.

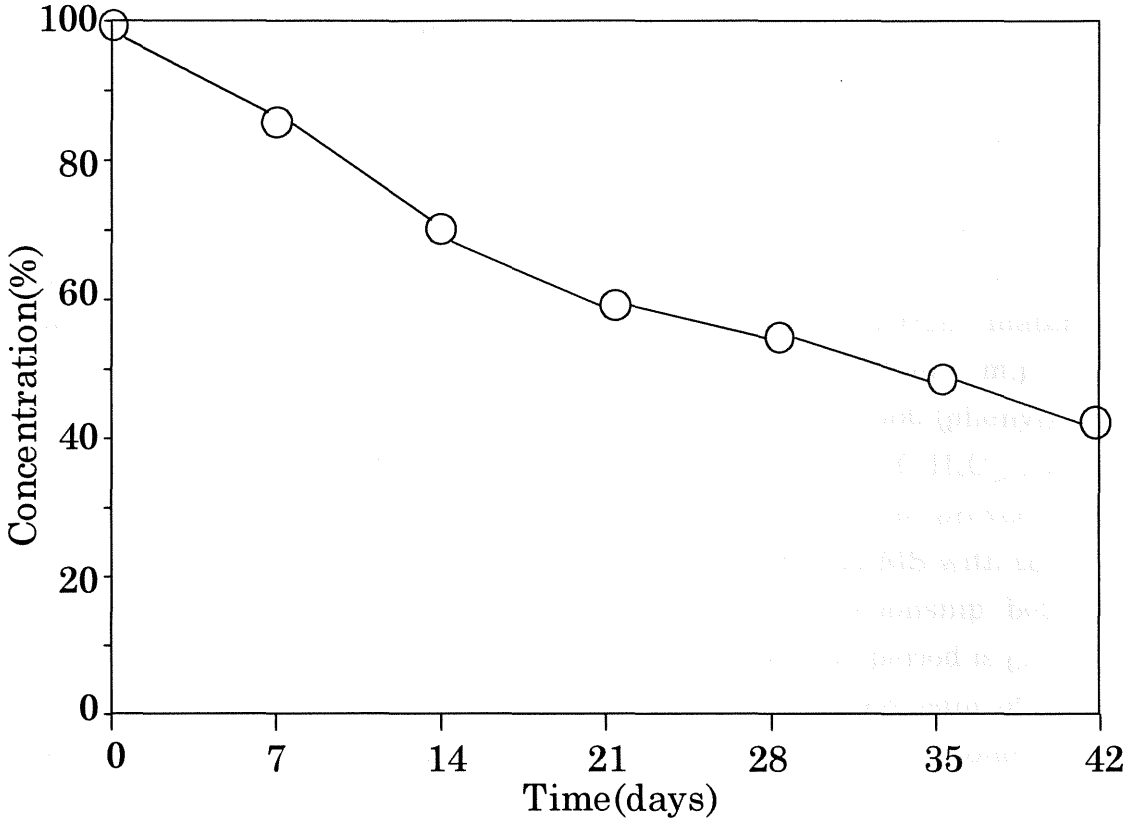


Fig.3-1 The degree of degradation of benzyl alcohol with time.

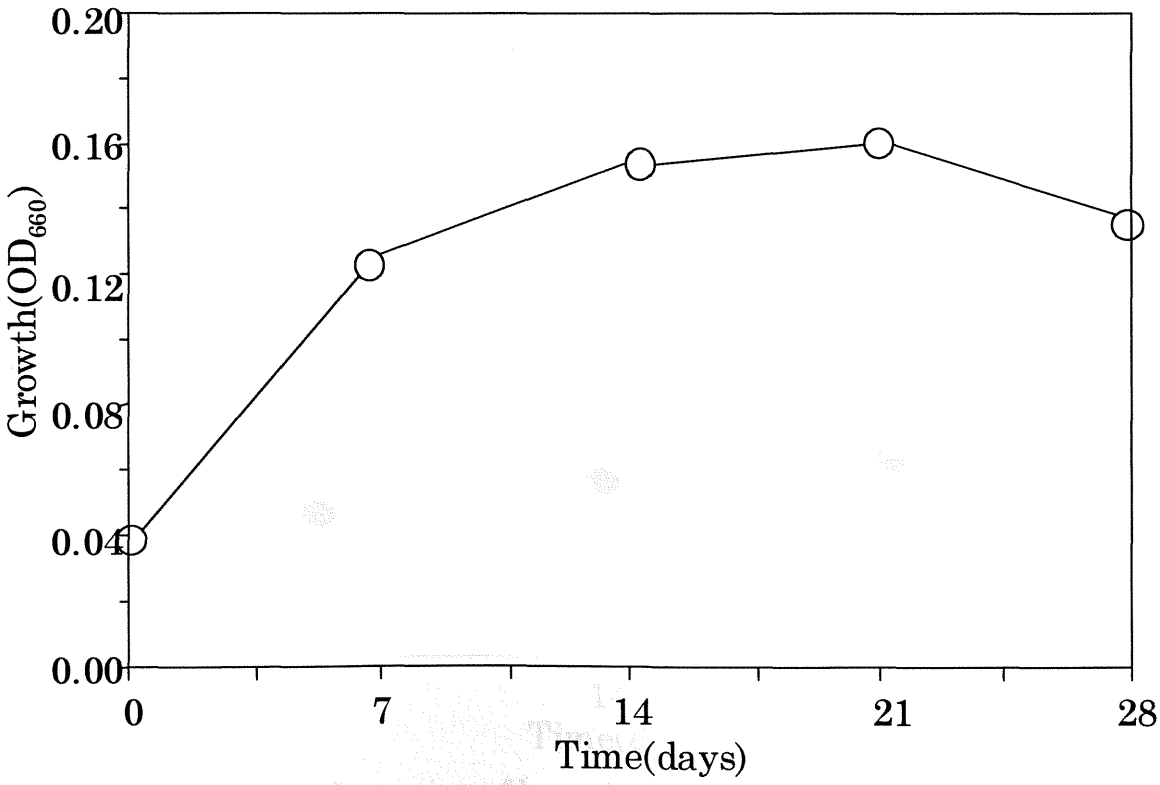


Fig.3-2 OD_{660} values of culture liquid with time.

2. Transformation of benzyl alcohol to salicyl alcohol

After culture for a month, culture liquids (5liters) were evaporated in a vacuum to light yellow materials, which were chromatographed by preparative TLC (Merck, Art 5715 Kieselgel 60 F_{254}) with a solvent system of $CHCl_3$-methanol-acetic acid (95:5:1). The band, R_f 0.4, was collected and stripped with methanol, and the solution was evaporated to dryness. The materials obtained were purified by another TLC to colorless materials (200mg). Physical properties of the metabolite were as follows. m.p. 86℃ (lit.[12] m.p. 86-87℃). IR υ_{max} (KBr) cm^{-1}: 2800-3200, (OH), 1600 (phenyl), 740 (*o*-substituted phenyl); EI-MS m/z: 124 (M^+, corresponding to $C_7H_8O_2$, 124.13), 106 (M^+-H_2O), 78 ($C_6H_6^+$). Consequently, this compound was proved to be identical with salicyl alcohol by comparing its m.p., IR and MS with those of an authentic sample. In Fig.3-3, the quantitative relationship between benzyl alcohol and salicyl alcohol during the bioconversion period is given. It shows that the quantity of salicyl alcohol increases with decreasing of benzyl alcohol. This preparation of salicyl alcohol from benzyl alcohol by a microorganism is presented.

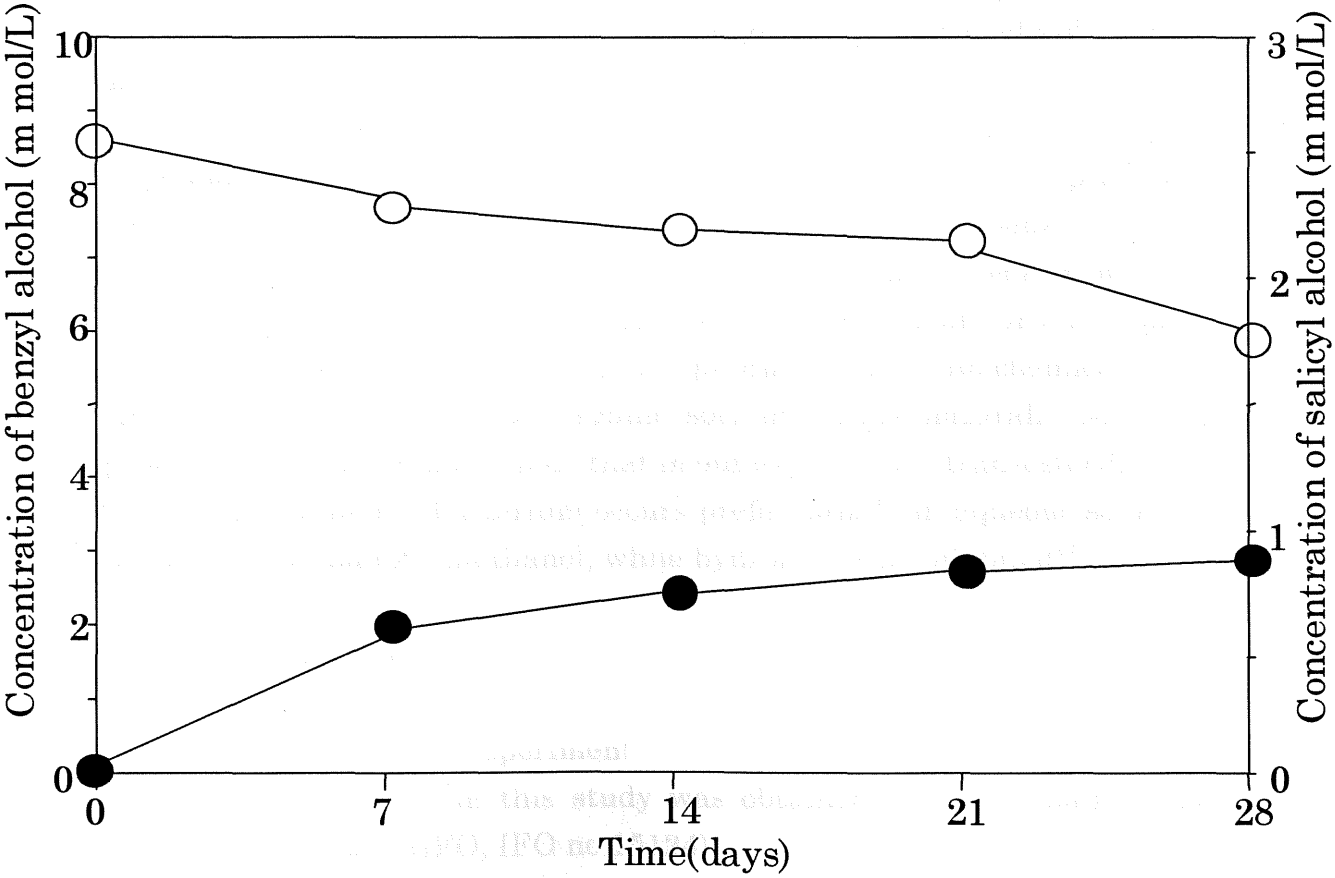


Fig.3-3 Transformation of benzyl alcohol to salicyl alcohol with time.
○, benzyl alcohol; ●, salicyl alcohol.

Chapter 4

Transesterification of Ethylparaben by Free and Entrapped Cells of *Burkholderia cepacia*

Ethylparaben was transesterified to methylparaben in the presence of methanol by *Burkholderia cepacia*. When the bacterium was incubated in water-methanol solutions of ethylparaben in the range of 0.01 to 2% methanol, the higher the content of methanol, the higher the conversion ratios of ethylparaben. If over 5% methanol was used, the bacterium could not survive and the reaction did not proceed. Transesterifications also proceeded with 1-propanol or benzyl alcohol instead of methanol, though the conversion ratios were low. Conversion ratios were found to be higher throughout the conversion period by entrapping the cells to Ca alginate or polyurethanes than for free cells. The results are presumably due to the higher stability of cells in 10% alcohol in which free cells could not survive. PU-3 and PU-6 are urethane prepolymers consisting of polyetherdiol and phenylisocyanate segments. PU-3 has a high polypropyleneglycol content, while PU-6 is high in polyethyleneglycol[22].

In the course of our investigations on the biotransformation of organic compounds by *Burkholderia cepacia*, we have reported that antiseptics such as parabens, methyl, ethyl, *n*-propyl and *n*-butyl *p*-hydroxybenzoates were almost completely degraded by the bacterium after three week incubation, and that *p*-hydroxybenzoic acid was identified as a metabolite in each case[15]. Recently, some studies have reported the production of useful chemicals *via* a transesterification reaction in organic solvents using naturally occurring lipase[23-25]. It is interesting to note that in our experiments transesterification of ethylparaben by the bacterium occurs preferentially in aqueous solutions containing less than 2% methanol, while hydrolysis was inhibited[29].

Materials and Methods

1. The strain used in this experiment

The bacterium used in this study was obtained from the Institute for Fermentation, Osaka (IFO, IFO no.15124).

2. Experimental equipments

The following equipment was used; IR: Hitachi 260-10, MS: Hitachi M-80B and HPLC: Shimadzu LC-6A.

3. Experimental procedures

Five hundred ml Erlenmeyer flasks containing 250ml of water was autoclaved for 20min. Ethylparaben was dissolved in this sterilized water at a concentration of 250ppm. When more than 1,000ppm of ethylparaben was used, the bacterium could not survive because of the toxicity. Methanol was then added at a concentration of 0.01, 0.1, 0.4, 0.8 or 2%. For 1-propanol and benzyl alcohol, the concentrations were 0.4 and 0.1%, respectively. A few loops of bacteria, subcultured on soybean casein digest agar medium for 2days at 30℃, were inoculated into the flasks before static incubation at 30℃.

4. Performance of HPLC

A mobile phase of 0.01M $NH_4H_2PO_4$-CH_3CN (65:35)(pH adjusted to 2.5 with H_3PO_4) was used for HPLC analysis of the incubated liquids. The column was a Shiseido Capcell Pak C_{18} SG-120, commercially packed with reversed-phase octadecylsilica (150mm × 6.0mm I.D.), through which the above mobile phase was run at a flow-rate of 1.0ml/min. The UV detector was operated at 254nm and samples of 10 μl were injected onto the column. The concentrations of the compounds were calculated according to the internal standard method. The incubated filtrates were directly subjected to HPLC analysis after incubation for the specified time.

5. Entrapping method of the bacterium

The entrapping of the bacterium was performed in the usual way. Cells subcultured on soybean casein digest agar medium for 2days at 30℃ (5.0g: wet weight) were suspended in 15ml of sterilized water, to which 40ml of 7.5% sodium alginate solution had been added. A 3% calcium chloride solution was then added dropwise with agitation for 30min at room temperature. The gel which formed was washed thoroughly with sterilized water, and the entrapped bacteria (110g: wet weight) were obtained. For PU-3 and PU-6, 2g of each were added with agitation to 4ml of the cell suspension until the gel formed.

Results and Discussion

1. Transesterification by free cells

As shown in Table 4-1 and Fig.4-1, ethylparaben was transesterified to methylparaben in the presence of 0.4% methanol after 3days incubation, and the relative concentration of methylparaben was 69.9% based on 229ppm. This is the concentration at which ethylparaben is completely transformed to methylparaben. The conversion ratio was found to be 80.2%. The conversion ratios were found to increase as the content of methanol increased from 0.01 to 2%, although the reaction velocity decreased. The concentration of *p*-hydroxybenzoic acid, a metabolite from hydrolysis, decreased remarkably as the content of methanol increased. Ethylparaben was transesterified to 1-propylparaben in the presence of 0.4% 1-propanol. It was also transesterified to benzylparaben in the presence of 0.1% benzyl alcohol although the conversion ratios were low. No changes in the concentration of ethylparaben were observed under the same conditions in the blank test. Cell growth observed as the OD_{660} value gradually increased during the fermentation. Metabolites were identified by means of IR and MS.

Table 4-1 Maximum concentrations and conversion ratios of ethylparaben transesterification with alcohols.

Support for entrapping	Alcohol	Fermentation period(days)	Maximum concentration(%)	Conversion ratio(%)
None (free cells)	Methanol(0.1%)	3	31.8	56.8
None (free cells)	Methanol(0.4%)	3	69.9	80.2
None (free cells)	Methanol(2.0%)	19	84.0	96.4
None (free cells)	1-Propanol(0.4%)	8	26.2	27.2
None (free cells)	Benzyl alcohol(0.1%)	10	1.4	1.9
Ca alginate	Methanol(10%)	4	78.3	95.6
Ca alginate	1-Propanol(10%)	20	35.8	85.4
Ca alginate	Benzyl alcohol(1%)	20	2.9	18.6
PU-3	Methanol(10%)	10	67.4	68.6
PU-3	1-Propanol(10%)	10	30.1	35.6
PU-3	Benzyl alcohol(1%)	7	1.8	2.5
PU-6	Methanol(10%)	5	78.4	79.9
PU-6	1-Propanol(10%)	5	61.7	65.3
PU-6	Benzyl alcohol(1%)	19	2.2	2.9

2. Transesterification by entrapped cells

A 250ppm ethylparaben solution with 10% methanol was incubated with the entrapped cells (7.5g: wet weight) and allowed to stand at 30℃. The reaction velocity was remarkably decreased due to ethylparaben toxicity when ethylparaben over 1,000ppm was used. Incubations were also carried out with 250ppm ethylparaben solution containing 10% 1-propanol or 1%

benzyl alcohol. Procedures for the incubation and administration of substrates and HPLC analyses were similar to those described above for free cells.

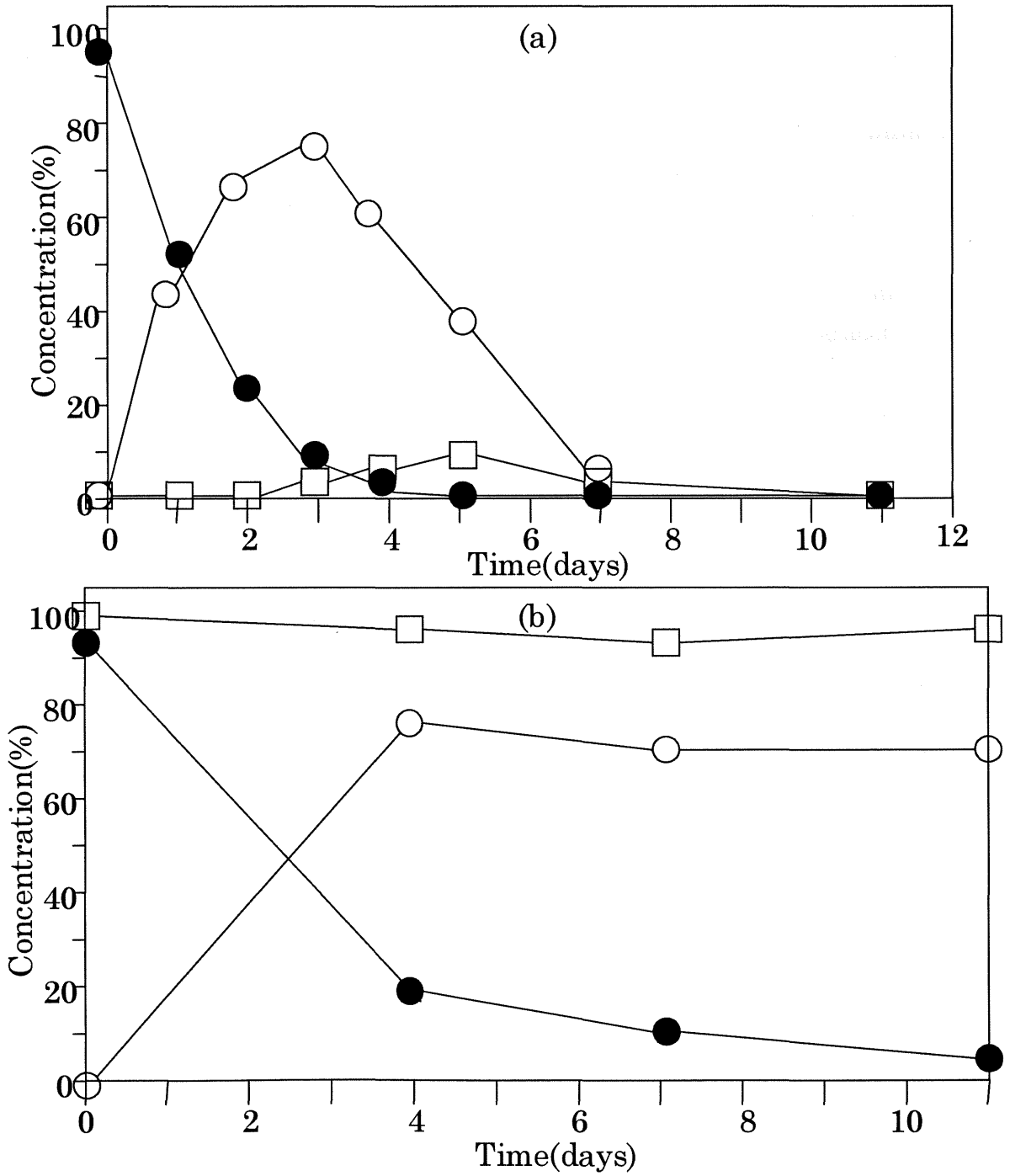


Fig.4-1 (a) Biotransformation of ethylparaben in the presence of 0.4% methanol over time, using free cells. Symbols: ●, ethylparaben; ○, methylparaben; □, *p*-hydroxybenzoic acid.
(b) Biotransformation of ethylparaben in the presence of 10% methanol over time, using entrapped cells (Ca alginate). Symbols: □, blank; ●, ethylparaben; ○, methylparaben.

As shown in Table 4-1 and Fig.4-1, conversion ratios were found to be higher throughout the conversion period by entrapping the cells compared to free cells. This is presumably because further hydrolysis of methylparaben was strongly inhibited. No changes in the concentration of ethylparaben were observed when examined under the same conditions as the blank test. This is most likely due to the protective effect of the gel towards the alcohols. Furthermore, high alcohol concentrations presumably support the inhibition of hydrolysis.

In the present study, it found that there were no living cells in the incubation medium during fermentation, and that repeated use of the entrapped cells was possible. From these results, it can be presumed that this reaction occurs *via* an extracellular esterase or lipase produced by the bacterium.

The authors gratefully acknowledge Professor A. Tanaka of Kyoto University for kindly donation the PU-3 and PU-6.

Chapter 5

Enantiomeric Resolution of 1-Phenyl-2-propanol by *Burkholderia cepacia*

Burkholderia cepacia hydrolyzed *rac*-1-phenyl-2-propyl acetate and propionate asymmetrically, affording (*R*)-(-)-1-phenyl-2-propanol and the ester of (*S*)-(+)-1-phenyl-2-propanol.

Stereospecific reactions by enzymes or microorganisms are useful in the preparation of a variety of optically active compounds. Especially in the preparation of chiral synthone, optical resolution of racemic compounds by microorganisms is so useful that many investigations have been reported[26,27].

In the course of our investigation on the biotransformation of organic compounds by *Burkholderia cepacia*, we have already reported that representative antiseptics, parabens[15], and a commonly used pesticide, carbaryl[20], were degraded by the bacterium. We also reported the regioselective hydroxylation of benzyl alcohol to salicyl alcohol by the bacterim[28]. This chapter reports the results of asymmetric hydrolysis of *rac*-1-phenyl-2-propyl acetate and propionate by the bacterium[30]. It is interesting that asymmetric hydrolysis occurs under simple conditions, in only water solutions of substrates by the bacterium.

Materials and Methods

1. The strain used in this experiment

The bacterium (IFO 15124) used in this experiment was given us by the IFO (Institute for Fermentation, Osaka).

2. Chemicals

Rac-1-phenyl-2-propanol, *rac*-1-phenyl-1-propanol and *rac*-1-phenyl-1-ethanol, and their (*R*),(*S*)-isomers (*e.e.* %$>$99) were purchased from Fluka Fine Chemicals. $[\alpha]_D^{20}$ of (*R*),(*S*)-isomers were as follows; (*R*)-1-phenyl-2-propanol, -41.0° ; (*S*)-1-phenyl-2-propanol, +41.1° ; (*R*)-1-phenyl-1-propanol, +46.9° ; (*S*)-1-phenyl-1-propanol, -46.8° ; (*R*)-1-phenyl-1-ethanol, +44.8° ; (*S*)-1-phenyl-1-ethanol, -44.9° . Acetylation and propionylation were done by

treating the chemicals with acetic anhydride or propionic anhydride in pyridine, respectively. The products were extracted with ethyl acetate and purified by preparative TLC on silica gel (Merck, Art 5715 Kieselgel 60 F_{254}) with a solvent system of hexane-ethyl acetate (8:2). The esterified products were almost 50% (*R*)-isomer and almost 50% (*S*)-isomer by HPLC.

3. Experimental procedures

In each case, a number of 500-ml Erlenmeyer flasks containing 250ml of water were autoclaved for 20min, then ester was added. A 200 μ g/ml solution of esters was used as a culture medium. A few loops of bacteria, cultured on SCD (soybean casein digest) agar medium, containing 1.5% peptone, 0.5% soybean peptone, 0.5% NaCl and 1.5% agar were inoculated into flasks, which were left for about seven days at 30℃.

After culture for a week, when almost half the esters were hydrolyzed, the incubated solution with cells was extracted with ethyl acetate. Evaporation of the solvent gave yellow materials, which were separated by preparative TLC on silica gel (Merck, Art 5715 Kieselgel 60 F_{254}) with a solvent system of hexane-ethyl acetate (8:2). A well-separated band, R_f: 0.8, was collected and stripped with methanol, then put on HPLC to measure the optical purity. Another well-separated band, R_f: 0.3, was collected and stripped with methanol, which was esterified with acetic anhydride. The reaction product was dissolved in methanol and was put on HPLC to measure the optical purity. No change in the concentration of esters and ratios of (*R*)-isomers and (*S*)-isomers were observed under the same conditions in the blank test.

4. Experimental equipments and analytical conditions

For HPLC analysis, the mobile phase, 0.01M $NH_4H_2PO_4$ (adjusted to pH 2.5 with H_3PO_4) - CH_3CN (6:4) was used. The column used was Shiseido Capcell Pak C_{18} SG-120 (150mm×6.0mm I.D.), commercially packed with reversed-phase octadecylsilica, through which the above mobile phase was run at a flow-rate of 1.0ml/min. The detection was done at 254nm and samples of 10 μ l were injected onto the column. The concentrations of the compounds were measured by the internal standard method.

HPLC for measurement of optical purity was done as follows. As the mobile phase, methanol was used. The column used was a Shiseido Ceramospher Chiral RU-1 (250mm×4.6mm I.D.), commonly used for the

separation of *rac*-compounds, through which methanol was run at a flow-rate of 1.0ml/min. The detection was done at 254nm and samples of 10 μ l were injected onto the column. The optical purities were measured by the peak area ratio. Retention times of (*R*)-isomers and (*S*)-isomers are shown in Table 5-1.

Table 5-1 Retention times of (*R*),(*S*)-1-phenyl-2-propyl, (*R*),(*S*)-1-phenyl -1-propyl and (*R*),(*S*)-1-phenyl-1-ethyl acetate and propionate.

Compound	Retention time of (*S*)-isomer (min)	Retention time of (*R*)-isomer (min)
1-Phenyl-2-propyl-acetate	4.5	5.0
1-Phenyl-2-propyl-propionate	4.4	5.0
1-Phenyl-1-propyl-acetate	7.0	8.8
1-Phenyl-1-propyl-propionate	6.9	8.5
1-Phenyl-1-ethyl-acetate	5.0	6.0
1-Phenyl-1-ethyl-propionate	4.6	5.5

Results and Discussion

1. Optical resolution of 1-phenyl-2-propanol

1-Phenyl-2-propanol, an important chiral synthone, was resolved optically by asymmetric hydrolysis of its acetate and propionate by the bacterium. After *rac*-1-phenyl-2-propyl acetate or propionate was incubated for a week with the bacterium, the (*S*)-(+)-ester and (*R*)-(-)-free alcohol were found to be almost at equal contents; about 50% toward the initial concentration of *rac*-compounds, suggesting no other reactions occur without hydrolysis. Optical purities of (*S*)-(+)-esters and (*R*)-(-)-free alcohols were 60-80% *e.e.* measured by HPLC.

2. Optical resolution of 1-phenyl-1-propanol

On the other hand, *rac*-1-phenyl-1-propanol was similarly resolved optically, though the optical purities were low.

3. Optical resolution of 1-phenyl-1-ethanol

In addition, *rac*-1-phenyl-1-ethanol was also resolved optically only when its propionate was used as the substrate. When its acetate was used as the substrate, however, *rac*-1-phenyl-1-ethanol was not resolved optically.

4. The numbers of the bacteria

The numbers of the bacteria were found to increase gradually during the bioconversion period, measured by the OD_{660} of the culture liquid. For instance, the OD_{660} of the culture liquid increased from 0.05 to 0.15 during the bioconversion period.

Conclusion

Rac-1-phenyl-2-propyl acetate and propionate were resolved optically to (*R*)-(-)-1-phenyl-2-propanol and (*S*)-(+)-1-phenyl-2-propyl acetate or (*S*)-(+)-1-phenyl-2-propyl propionate. Similarly, *rac*-1-phenyl-1-propyl acetate and propionate were resolved optically to (*R*)-(+)-1-phenyl-1-propanol and (*S*)-(-)-1-phenyl-1-propyl acetate or (*S*)-(-)-1-phenyl-1-propyl propionate. On the other hand, *rac*-1-phenyl-1-ethyl propionate was resolved optically to (*R*)-(+)-1-phenyl-1-ethanol and (*S*)-(-)-1-phenyl-1-ethyl propionate, while *rac*-1-phenyl-1-ethyl acetate was not resolved optically. Hydrolysis ratios and optical purities are shown in Table 5-2.

Esterase produced by the bacterium was found to hydrolyze (*R*)-isomers of esters stereoselectively. In the case of *rac*-1-phenyl-1-ethyl acetate, however, stereoselective hydrolysis did not occur.

Table 5-2 Asymmetric hydrolysis of *rac*-1-phenyl-2-propyl, *rac*-1-phenyl-1-propyl and *rac*-1-phenyl-1-ethyl acetate or propionate.

Substrate	Hydrolysis ratio (%)[a]	*e.e.* (%) of (*R*)-free alcohol	*e.e.* (%) of (*S*)-ester
rac-1-Phenyl-2-propyl-acetate	50.1	78.3	70.7
rac-1-Phenyl-2-propyl-propionate	46.9	64.0	78.9
rac-1-Phenyl-1-propyl-acetate	56.0	25.4	44.3
rac-1-Phenyl-1-propyl-propionate	50.8	Not measured	14.8
rac-1-Phenyl-1-ethyl-propionate	45.4	80.3	79.6

[a] Molar ratio of free alcohol to initial *rac*-ester.

Summary

1. The bacterial strain used in this study was isolated from benzalkonium chloride solution. It was identified as *Burkholderia cepacia* according to Bergy's manual at The Research Foundation for Microbial Disease of Osaka University. It is now preserved as IFO No.15124 strain at the Institute for Fermentation Osaka.
2. Parabens were completely degraded in three weeks by the strain. We isolated and identified *p*-hydroxybenzoic acid and protocatechuic acid as metabolites. It was clarified that the strain degraded many organic residues.
3. To control the reaction, we tried to transform organic residues to useful materials.
   1) Benzyl alcohol was biotransformed to salicyl alcohol stoichiometrically. Salicyl alcohol was accumulated because of relatively little further degradation.
   2) D-Tryptophan was accumulated with L-specific digestion of tryptophan by the strain.
   3) *Rac*-1-phenyl-2-propyl acetate was hydrolyzed (*R*)-selectively to (*R*)-(-)-1-phenyl-2-propanol and (*S*)-(+)-1-phenyl-2-propyl acetate by the bacteria. *Rac*-1-phenyl-1-ethyl propionate was hydrolyzed (*R*)-selectively to (*R*)-(+)-1-phenyl-1-ethanol and (*S*)-(-)-1-phenyl-1-ethyl propionate by the bacteria.
4. We obtained the following further experimental results; the reaction efficiencies were sometimes increased by immobilization of the strain.
   1) Encapsulated cells of the strain by polyacrylamide degraded carbaryl rapidly than the case of free cells. Furthermore, second-used encapsulated cells also degraded carbaryl rapidly than the case of free cells.
   2) We clarified the strain catalyzed transesterification in the presence of alcohol. The strain catalyzed transesterification of substrates adding excess alcohol. It was clarified that we could change the type of ester using this reaction. It is noteworthy that using immobilized cells, we could use higher contents of alcohol and could have higher conversion ratio.

The strain was formerly classified as *Pseudomonas cepacia*. Actually the strain has spherical shape like an onion and hard to say rod. Also in this meaning the strain is interesting one in environmental bacteria and unique target to research. We know the strain degrade many toxic chemicals and product useful compounds. To investigate the enzymes of the strain, this research will be accelerated furthermore.

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